

APPEnDIX 2

## Enhanced Apoptosis of B and T Lymphocytes in TAF<sub>II</sub>105 Dominant-negative Transgenic Mice Is Linked to Nuclear Factor-κB\*

Received for publication, January 23, 2002, and in revised form, February 13, 2002  
Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.M200696200

Antonina Silkov‡§, Orit Wolstein‡, Idit Shachar¶, and Rivka Dikstein‡||

From the Departments of ‡Biological Chemistry and ¶Immunology, Weizmann Institute of Science, Rehovot 76100, Israel

The general transcription factor TFIID is composed of the TATA-binding protein (TBP) and 12–14 TBP-associated factors (TAF<sub>II</sub>s). Some TAF<sub>II</sub>s act as bridges between transcription activators and the general transcription machinery through direct interaction with activation domains. Although TAF-mediated transcription activation has been established, there is little genetic evidence connecting it to binding of an activator. TAF<sub>II</sub>105 is a substoichiometric subunit of transcription factor IID highly expressed in B lymphocytes. In this study, we examined the physiological role of TAF<sub>II</sub>105 and its mechanism of action *in vivo* by expressing two forms of dominant-negative mutant TAF<sub>II</sub>105 in mice. We show that TAF<sub>II</sub>105 has a pro-survival role in B and T lymphocytes, where the native protein is expressed. In addition, TAF<sub>II</sub>105 is important for T cell maturation and for production of certain antibody isotypes. These phenotypic alterations were absent in mice expressing a dominant-negative mutant that lacks one of the domains mediating p65/RelA binding *in vitro*. These findings provide support to the notion that interaction between the activator and TAF is important for their function *in vivo*.

The basal transcription factor TFIID is a multisubunit complex consisting of TATA-binding protein (TBP)<sup>1</sup> and associated factors (TAF<sub>II</sub>s). TAF<sub>II</sub>s display multiple functions related to transcription regulation (1, 2). They are required for recognition and binding to core promoter elements such as the initiator and the downstream promoter element (3–8). In addition, certain TAF<sub>II</sub>s interact with activation domains of transcription factors, interactions that were found to be essential for transcription activation by activators *in vitro* (9). The importance of TAF<sub>II</sub>s in the transcription activation process has also been supported by several studies in cultured cells (10–16) and by genetic experiments in *Drosophila* (17, 18).

Despite the significant progress that has been made in the

characterization of TAF<sub>II</sub>s at the biochemical level, little is known about the physiological relevance of these studies or about the specific functions of individual TAF subunits in biological processes involving transcription regulatory programs. An important issue concerning the mechanism of TAF-mediated transcription activation is whether direct contact between activators and TAF<sub>II</sub>s is indeed essential for transcription *in vivo* and what is the consequence of activator-TAF interaction *in vivo*. Examination of activator-TAF connection is of particular interest because activators have multiple potential coactivator targets within the transcription machinery.

Studies in yeast carrying mutations in individual TAF<sub>II</sub> subunits indicated that some TAF<sub>II</sub>s are required for transcription of the majority of class II genes and others for transcription activation of only subsets of genes (19–28). Interestingly, inactivation of certain individual TAF<sub>II</sub>s dramatically affects the stability and integrity of the entire TFIID complex *in vivo* (5, 20, 22, 24, 27–29), complicating the understanding of the function of the mutant subunit. Furthermore, some of the TAF<sub>II</sub>s are shared between TFIID and other complexes such as yeast SAGA and human PCAF and TFTC (30, 31); thus, a phenotypic alteration observed with these mutant TAF<sub>II</sub>s may be linked to any of the complexes containing TAF.

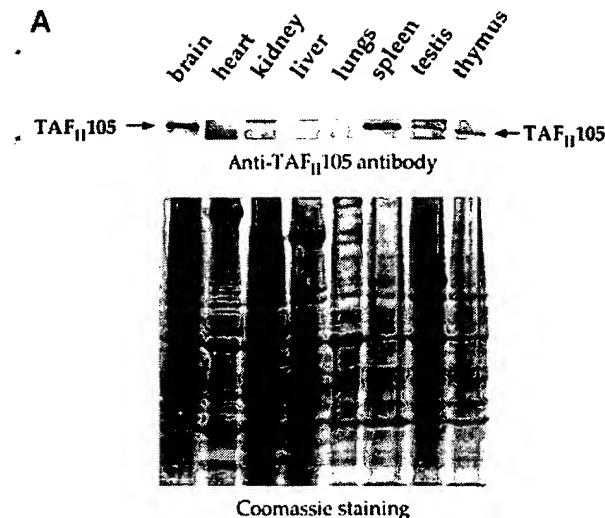
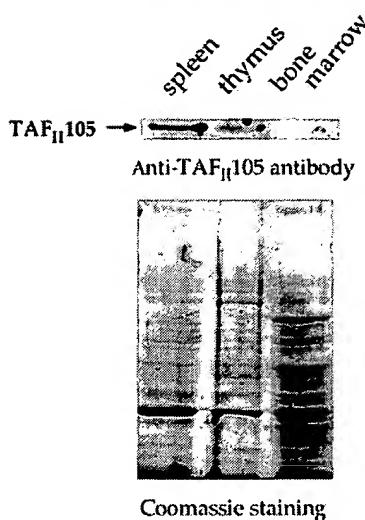
TAF<sub>II</sub>105 is a member of the human TAF<sub>II</sub>135 and *Drosophila* TAF<sub>II</sub>110 family of TAF<sub>II</sub>s, which has several unique properties. Unlike the core TAF<sub>II</sub>s that are expressed in most cell lines at similar levels, TAF<sub>II</sub>105 expression is regulated. It is more abundant in the TFIID complex of human B lymphocytes than in non-B cells (32). Consistent with this expression pattern, TAF<sub>II</sub>105 was found to be involved in transcription activation by p65/RelA, a member of the NF-κB family (15), and OCA-B (14); both are required for lymphocytic gene expression. TAF<sub>II</sub>105 appears to be present only in a small fraction of TFIID complexes and therefore might be involved in transcription of a relatively small subset of genes. The C-terminal domain of human TAF<sub>II</sub>105 and TAF<sub>II</sub>130 and *Drosophila* TAF<sub>II</sub>110 is highly conserved and has interaction surfaces with other TAF<sub>II</sub>s, implicating it in the assembly of the TFIID complex. The N terminus of TAF<sub>II</sub>105 is more variable and contains binding sites for the activation domains of p65/RelA and OCA-B (14, 15). Because TAF<sub>II</sub>105 is found only in a small portion of TFIID complexes and may be required for transcription of a small subset of genes, but is homologous to one of the core subunits, it has the potential to be a good candidate for genetic analysis in mammals. However, recent data obtained from the Human Genome Project revealed another gene on chromosome 4 encoding a close homolog of TAF<sub>II</sub>105 (accession number AC017007). The previously identified gene is encoded by chromosome 18 (accession number Y09321). The existence of an expressed sequence tag clone identical to the chromosome 4 gene and the fact that a sequence of one of the peptides derived from the TAF<sub>II</sub>105 protein more closely resembles the chromo-

\* This work was supported by the Israel Cancer Research Foundation and by institutional internal grants from the Henri and Francoise Glasberg Foundation, Mr. and Mrs. Lon Morton, and the Foundation of Raphael and Regina Levy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Dept. of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032.

¶ To whom correspondence should be addressed: Dept. of Biological Chemistry, Weizmann Institute of Science, 2 Hertzl St., Rehovot 76100, Israel. Tel.: 972-8-934-2117; Fax: 972-8-934-4118; E-mail: rivka.dikstein@weizmann.ac.il.

<sup>1</sup> The abbreviations used are: TBP, TATA-binding protein; TAF<sub>II</sub>, TFIID-associated factor; TAF, TBP-associated factor; NF-κB, nuclear factor-κB; KLH, keyhole limpet hemocyanin; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter.

**A****B**

**FIG. 1. Expression pattern of endogenous TAF<sub>II</sub>105 in mouse tissues.** *A:* upper panel, protein extract was prepared from mouse tissues as described under "Materials and Methods" and analyzed by Western blotting using affinity-purified anti-TAF<sub>II</sub>105 antibody. Lower panel, the same amounts of protein extracts were examined by Coomassie Blue staining. *B:* comparison of TAF<sub>II</sub>105 expression in the indicated lymphoid tissues by Western blotting and Coomassie Blue staining.

some 4 gene (data not shown) strongly suggest that this gene is also expressed and may display some redundant functions with the gene encoded by chromosome 18. Recently, mice deficient in TAF<sub>II</sub>105 were generated; the females of these mice were found to be sterile due to defects in folliculogenesis, but no obvious phenotypic alterations in lymphoid organs were found (33).

In this study, we addressed the physiological role and the mechanism of action of TAF<sub>II</sub>105 *in vivo* by generating animal models expressing dominant-negative mutant forms of TAF<sub>II</sub>105 that are likely to inhibit the function of TAF<sub>II</sub>105 proteins encoded by different genes. The results revealed that TAF<sub>II</sub>105 is essential for the survival of B and T lymphocytes, where the native protein is highly expressed. This function of TAF<sub>II</sub>105 is dependent on a domain involved in the interaction with the NF- $\kappa$ B protein p65/RelA *in vitro*, suggesting that activator-TAF interaction is important for the function of TAF<sub>II</sub>s in the transcription process *in vivo*.

## MATERIALS AND METHODS

**Construction of Transgenes.**—For the generation of a lymphoid-specific TAF<sub>II</sub>105AC expression plasmid, a TAF<sub>II</sub>105 fragment encoding amino acids 1–552 fused to a hemagglutinin tag and a  $\beta$ -globin intron was ligated into the *Hpa*I and *Xba*I sites of the pTDK vector (kindly provided by Dr. M. van Lohozien). This vector contains a duplicated immunoglobulin heavy chain enhancer upstream of the *pim-1* promoter and a murine leukemia virus long terminal repeat within the 3'-region (36). The fragment containing coding and regulatory sequences was excised from the vector by *Sal*I digestion. No expression of the transgene was observed in lymphoid organs of the transgenic mice (data not shown).

To generate transgenes ubiquitously expressing TAF<sub>II</sub>105 mutants, the previously described plasmids pCMV-TAF<sub>II</sub>105AC-(1–552) and pCMV-TAF<sub>II</sub>105AC-( $\Delta$ 452–472) were used (35). The plasmids were linearized by *Afl*III/*Xba*I digestion before microinjection.

**Generation of Transgenic Mice.**—The DNA fragments were injected into the male pronucleus of (C57BL/6  $\times$  Balb/c)F<sub>1</sub> embryos. Transgenic mice among the progeny were identified by Southern blot analysis. Mice were bred and maintained under standard conditions in the Weizmann Institute of Science Transgenic Facility.

**Immunization of Mice.**—Mice were immunized intraperitoneally at 6–10 weeks of age with 50  $\mu$ g of keyhole limpet hemocyanin (KLH) (Calbiochem-Novabiochem) in complete Freund's adjuvant (Difco).

**Southern Blot Analysis.**—For the detection of the transgene in the mouse genome, 10  $\mu$ g of total genomic DNA from each mouse was digested with *Eco*RI, separated on 1% agarose gels at 50 V, and transferred to GeneScreen Plus nylon membrane (PerkinElmer Life Sciences). Filters were hybridized at 65 °C overnight with a <sup>32</sup>P-labeled fragment of TAF<sub>II</sub>105AC (C-terminal truncated mutant). The probe was prepared using the Rediprime random primer labeling system (Amersham Biosciences, Buckinghamshire, United Kingdom).

For the identification of A20 PCR products, PCR products were run on 1.2% agarose gel and transferred to nylon membranes. The membranes were hybridized at 37 °C for 2 h with a <sup>32</sup>P-labeled oligonucleotide (TTGACAGAAAGTGTCCAGGCT). The oligonucleotide was labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase.

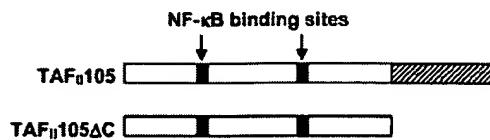
**RT-PCR and Real-time Quantitative RT-PCR.**—Total RNA was extracted from spleen and thymus using Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's instructions. RNA preparations were treated with RQ1 DNase I (Promega) to avoid contamination with genomic DNA. For reverse transcription, 1  $\mu$ g of total RNA was incubated at 42 °C in the presence of avian myeloblastosis virus reverse transcriptase (Promega), avian myeloblastosis virus reverse transcriptase buffer (Promega), 10 pm reverse-specific primer, 0.1 M dithiothreitol, 2  $\mu$ l of 10 mM dNTP, and 40 units of RNasin (Promega) in a total volume of 30  $\mu$ l.

RT-PCR analysis for the presence of transgenic mRNA was performed with total RNA using primers that spanned the intron of the transgene so that a PCR product obtained from spliced mRNA differed in size from a product resulting from plasmid or genomic DNA amplification. Quantitative PCR was performed in 20- $\mu$ l glass capillary tubes using a LightCycler system (Roche Molecular Biochemicals) equipped with a thermal cycler and a real-time detector of fluorescence. 2  $\mu$ l of cDNA was amplified specifically using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

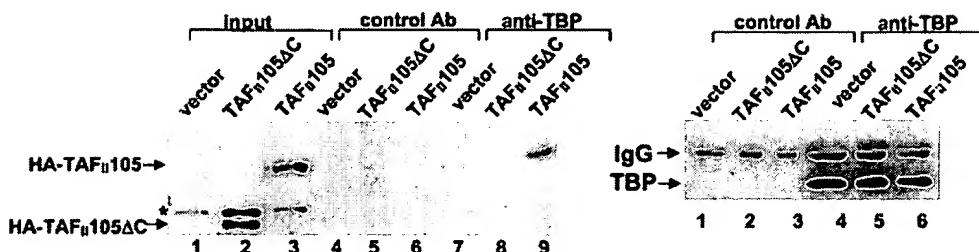
The oligonucleotides used for RT-PCR are as follows: for the detection of transgenic mRNA, transgene forward (CAGCCTTCAGGAG-GCAATGA) and transgene reverse (TAGCCAGAAAGTCAGATGCTC); and for the analysis of A20 expression, A20 forward (CGGAAAGCTG-TGAAGATACGAGAG), A20 reverse (TTCCAGTCCGAGTGTGCGT-GC), GAPDH forward (GCCATCAACGACCCCTTCAT), and GAPDH reverse (ITCACACCCATCACAAACAT).

**Protein Analysis.**—Total cell extracts from mouse tissues were prepared as described (51). Cell extracts from transfected 293T cells or from splenocytes were prepared by lysing the cells in buffer containing 50 mM Tris (pH 7.9), 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin. Nuclei and cell debris were removed by centrifugation. For immunoprecipitation, 1  $\mu$ l of either anti-TBP or anti-invertase (control) serum was used. Proteins were separated by SDS-PAGE, transferred to Protran BA83 nitrocellulose membrane (Schleicher & Schüll), and incubated with the indicated primary antibodies. The blot was then incubated with goat horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Jackson Immuno-Research Laboratories, Inc., West Grove, PA), followed by enhanced

A.



B.



**FIG. 2. Characterization of dominant-negative mutant TAF<sub>II</sub>105.** *A*, schematic representation of wild-type and dominant-negative mutant TAF<sub>II</sub>105. *B*, TAF<sub>II</sub>105, but not TAF<sub>II</sub>105ΔC, is associated with the TFIID complex. 293T cells were transfected with plasmids encoding hemagglutinin (HA)-tagged TAF<sub>II</sub>105 and TAF<sub>II</sub>105ΔC. Total cell extracts were prepared 48 h after transfection and incubated with anti-TBP (lanes 7-9) or anti-invertase (lanes 4-6) polyclonal antibody (Ab) bound to protein A-Sepharose beads. Bound proteins were eluted and analyzed by Western blotting using anti-hemagglutinin monoclonal antibody (left panel) and anti-TBP antibody (right panel). The asterisk corresponds to a non-specific band.

chemiluminescent staining using SuperSignal chemiluminescent substrate (Pierce).

**Flow Cytometry**—Single cell suspensions from spleen and thymus were prepared by crushing the organs in RPMI 1640 medium without serum, followed by hypotonic lysis of erythrocytes. Freshly isolated cells were incubated on ice for 0.5 h with the following antibodies: anti-B220 (CyChrome), anti-CD4 (PE), and anti-CD8a (CyChrome) (all from BD PharMingen). Apoptotic cells were detected using an ApoScreen fluorescein isothiocyanate-annexin V apoptosis kit (Southern Biotechnology Associates, Inc., Birmingham, AL). Stained cells were analyzed on a FACScan using Cell Quest software (both from BD PharMingen).

**Enzyme-linked Immunosorbent Assays**—For enzyme-linked immunosorbent assays, 96-well plates (Nalge Nunc International, Naperville, IL) were coated overnight at 4 °C with 5 µg/ml KLH solution in phosphate-buffered saline. After removing the KLH solution, the plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20. Serial dilutions from the sera of immunized mice were added and incubated for 2 h at room temperature. After extensive washes, the plates were incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat antibodies against different classes of mouse immunoglobulins (Clonotyping System HRP, Southern Biotechnology Associates, Inc.). The plates were washed again four times. ABTS solution (Sigma) was added, and the enzymatic reaction was quantitated in a microplate reader. The levels of anti-KLH Ig subclasses (at a serum dilution of 1:200) were determined 1 and 2 weeks after immunization. Results are expressed as  $A_{405}$  values.

**Chromatin Immunoprecipitation Assay**—This assay was performed on the basis of a previously published protocol with some modifications (52). Daudi B cells ( $10^8$ ) were cross-linked *in vivo* with 1% formaldehyde for 10 min at room temperature. Cells were washed once with cold phosphate-buffered saline, once with 2.5 ml of buffer I (10 mM Hepes (pH 6.5) 10 mM EDTA, 0.5 mM EGTA, and 0.25% Triton X-100), and once with buffer II (10 mM Hepes (pH 6.5) 1 mM EDTA, 0.5 mM EGTA, and 200 mM NaCl). Cells were resuspended in 1 ml of lysis buffer (50 mM Tris (pH 8.1) 10 mM EDTA, 1% SDS, 0.8 µg/ml pepstatin A, 0.6 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and sonicated 10 times for 10 s each time. The extract was then clarified by centrifugation for 15 min in a microcentrifuge at 4 °C and diluted 10-fold with dilution buffer (20 mM Tris (pH 8.1), 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100) to yield the solubilized chromatin. Next, salmon sperm DNA (50 µg/ml), tRNA (100 µg/ml), and bovine serum albumin (1 mg/ml) were added; and the extract was precleared by

addition of 50 µl of 50% protein A-Sepharose suspension beads/ml and incubation for 30 min on a rotator wheel. After 15 min of centrifugation at 4 °C, the extract was transferred to a new tube. A sample of 5 µl of the soluble extract was analyzed on 1% agarose gel to confirm an average size of 1-kb DNA and the relative amount of the input material for each sample. For immunoprecipitation, 1 µl of anti-TBP, anti-TAF<sub>II</sub>105, anti-p65, or control sera was added to 0.5 ml of the soluble chromatin (corresponding to 5–10  $\times 10^6$  cells) and incubated from 4 h to overnight at 4 °C. After centrifugation, the extracts were transferred to a new tube containing 25–30 µl of 50% protein A-Sepharose suspension and incubated at 4 °C for 1–2 on the rotator wheel. The beads were then washed sequentially with 150 mM NaCl + TSE buffer (TSE buffer: 20 mM Tris (pH 8.1), 0.1% SDS, 2 mM EDTA, 1% Triton X-100, and 150 or 500 mM NaCl), 500 mM NaCl + TSE buffer, and buffer III (10 mM Tris (pH 8.1), 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, and 1 mM EDTA) and three times with TE buffer (10 mM Tris (pH 8) and 1 mM EDTA). The immune complexes were then eluted from the Sepharose beads by incubating the beads three times with 100 µl of elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>, and 20 µg/ml glycogen) for 10 min each time. The combined eluates were heated at 65 °C for 4 h to reverse the formaldehyde cross-links. The eluates were extracted once with phenol/chloroform and once with chloroform, precipitated with ethanol, and resuspended in 20 µl of TE buffer. 2 µl were used for PCR. The primers used for the A20 promoter were 5'-CAG CCC GAC CCA GAG AGT CAC-3' and 5'-CGGGCTCCAAGCTCGCTT-3'.

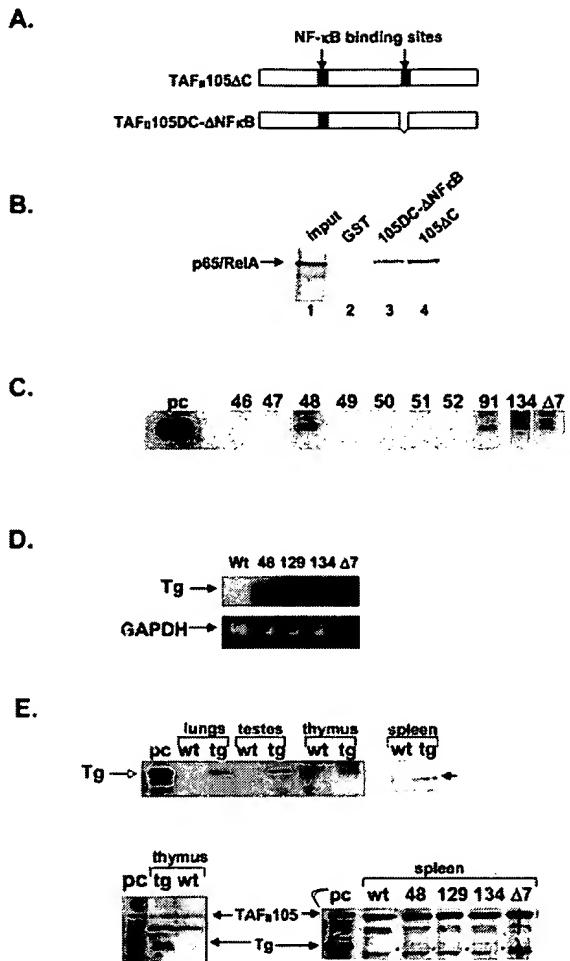
## RESULTS

**Expression of TAF<sub>II</sub>105 in Mouse Tissues**—As a first step toward understanding the biological role of TAF<sub>II</sub>105, we determined the expression pattern of the TAF<sub>II</sub>105 protein in mouse tissues. The TAF<sub>II</sub>105 protein was found in brain, testis, spleen, and thymus and was undetectable in lungs, liver, kidney, and heart (Fig. 1A). The presence of TAF<sub>II</sub>105 in the lymphoid organs is consistent with previous results showing high levels of expression of TAF<sub>II</sub>105 in mature B cell lines (32). TAF<sub>II</sub>105 was not expressed at detectable levels in bone marrow, where B and T cells originate (Fig. 1B). The significant expression of TAF<sub>II</sub>105 at the mature stage of lymphocyte development suggests that it may play a role in immunological functions associated with differentiated B and T cells.

**Generation of Mouse Lines Expressing Dominant-negative Mutants of TAF<sub>II</sub>105**—To analyze the function of TAF<sub>II</sub>105 and the physiological importance of its interaction with the p65/RelA member of the NF- $\kappa$ B family, we set out to generate transgenic mice expressing dominant-negative mutant forms of this protein lacking the conserved C-terminal domain involved in interaction with other TAF<sub>II</sub>s (data not shown). When a C-terminal truncated mutant (TAF<sub>II</sub>105 $\Delta$ C) or a full-length TAF<sub>II</sub>105 protein was expressed in cultured cells, TAF<sub>II</sub>105 $\Delta$ C did not assemble into the TFIID complex, whereas the full-length protein did (Fig. 2B), indicating that TAF<sub>II</sub>105 $\Delta$ C is either loosely or not associated with TFIID. Similarly, TAF<sub>II</sub>130 C-terminal domain was recently reported to be important for assembly into the TFIID complex (34). Because TAF<sub>II</sub>105 $\Delta$ C binds to the p65/RelA activation domain similarly to the full-length protein (15), expression of this mutant in cells would compete with endogenous TAF<sub>II</sub>105 protein for binding with p65/RelA, but not with TFIID, thus exerting a dominant-negative effect. Previous experiments in cultured cells showed that TAF<sub>II</sub>105 $\Delta$ C acts as a specific inhibitor of TAF<sub>II</sub>105 function, as it has an effect similar to depletion of the endogenous protein by antisense expression (15). Furthermore, a C-terminal truncation of *Drosophila* TAF<sub>II</sub>110, a homologous subunit of TAF<sub>II</sub>105, displayed a phenotype that is identical to deletion of the TAF<sub>II</sub>110 gene (18). In view of the high degree of homology between the human and mouse genomes and the recent finding that two different human chromosomes encode highly related homologs of TAF<sub>II</sub>105 (see the Introduction), it is likely that TAF<sub>II</sub>105 $\Delta$ C would inhibit the function of both.

To generate the transgenic mice, we used expression plasmids encoding TAF<sub>II</sub>105 $\Delta$ C and TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B (Fig. 3A). TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B has an additional internal deletion of 18 amino acids spanning the C-terminal NF- $\kappa$ B-binding site within TAF<sub>II</sub>105 (35). Consistent with the presence of only one NF- $\kappa$ B-binding site, TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B bound only 40% of p65/RelA compared with TAF<sub>II</sub>105 $\Delta$ C (Fig. 3B).

Considering the possible role of TAF<sub>II</sub>105 in the immune system, our initial attempts were to achieve targeted expression of the dominant-negative mutant in lymphoid tissues using a lymphoid-specific expression vector (36). Although we have obtained 12 TAF<sub>II</sub>105 $\Delta$ C transgenic lines, we failed to detect TAF<sub>II</sub>105 $\Delta$ C RNA or protein expression in lymphoid tissues of these mice (data not shown). Therefore, we used another expression vector driven by the cytomegalovirus promoter, which is supposed to direct ubiquitous expression of the transgene. Among 10 transgenic lines carrying TAF<sub>II</sub>105 $\Delta$ C and eight lines carrying TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B transgenes in their genome (Fig. 3C and data not shown), only five and three lines, respectively, expressed transgenic mRNA and protein (Fig. 3, D and E). It should be noted that the level of expression of the transgene in lymphoid tissues was low and in all cases was below the level of expression of endogenous TAF<sub>II</sub>105 (Fig. 3E). The expression level of TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B (line  $\Delta$ 7) was higher than that of TAF<sub>II</sub>105 $\Delta$ C (Fig. 3E, lower panel), thus enabling us to compare their phenotypes. Furthermore, the level of expression of TAF<sub>II</sub>105 $\Delta$ C declined with generations, and variable levels of expression between individuals within the same line were observed. These observations may be explained by a possible toxic effect of TAF<sub>II</sub>105 $\Delta$ C. Transgenic mice, expressing the low level of TAF<sub>II</sub>105 $\Delta$ C protein, were viable, but more prone to microbial infections, occasionally emerging in transgenic facility (*Klebsiella pneumoniae*, *Micrococcus* spp., *Pseudomonas aeruginosa*, etc.). Some of the female mutant animals were sterile. The results of the subsequent experiments were reproducible in three independent lines ex-



**FIG. 3. Generation of transgenic mice expressing dominant-negative mutants of TAF<sub>II</sub>105.** *A*, shown is a schematic representation of TAF<sub>II</sub>105 dominant-negative mutants used for generation of transgenic mice. *B*, show is the interaction between TAF<sub>II</sub>105 $\Delta$ C or TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B and the NF- $\kappa$ B protein p65/RelA. Equal amounts of TAF<sub>II</sub>105 $\Delta$ C and TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B fused to glutathione *S*-transferase (GST) were purified from bacteria with glutathione-Sepharose beads and used for binding assays with <sup>35</sup>S-labeled p65/RelA protein. As a control for binding specificity, glutathione *S*-transferase protein was analyzed in a similar manner. *input* represents 10% of the labeled protein used for the binding. *C*, Southern blot analysis was carried out with a transgene-specific probe, showing integration of the transgenes into the mouse genome. Line  $\Delta$ 7 is transgenic for TAF<sub>II</sub>105 $\Delta$ C- $\Delta$ NF $\kappa$ B. The other lines are transgenic for the TAF<sub>II</sub>105 $\Delta$ C mutant. *D*, upper panel, RNA was extracted from splenocytes and used for RT-PCR analysis with a transgene (*Tg*)-specific primer set, followed by hybridization with a transgene-specific probe, showing mRNA expression of the transgenes. Lower panel, control RT-PCR was carried out with GAPDH primers. *E*, upper panel, Western blot analysis was carried out with anti-hemagglutinin antibody, showing expression of the transgene in mouse tissues from line 48. Lower panel, Western blot analysis was performed with anti-TAF<sub>II</sub>105 antibody, showing expression of the transgene in spleens from different transgenic lines. Note the low level of expression of the transgene (indicated by asterisks) compared with endogenous TAF<sub>II</sub>105. *pc*, positive control representing extract from human 293T cells transiently transfected with the TAF<sub>II</sub>105 $\Delta$ C transgene; *Wt/wt*, wild-type.

pressing the transgene, ruling out the possibility of integration position effect.

**Increased Levels of Apoptosis in Lymphocytes from TAF<sub>II</sub>105 $\Delta$ C Transgenic Mice**—To determine the effect of TAF<sub>II</sub>105 $\Delta$ C expression on the immune system, we analyzed B and T lymphocytes in spleen and bone marrow and found no significant difference in the relative number of these popula-

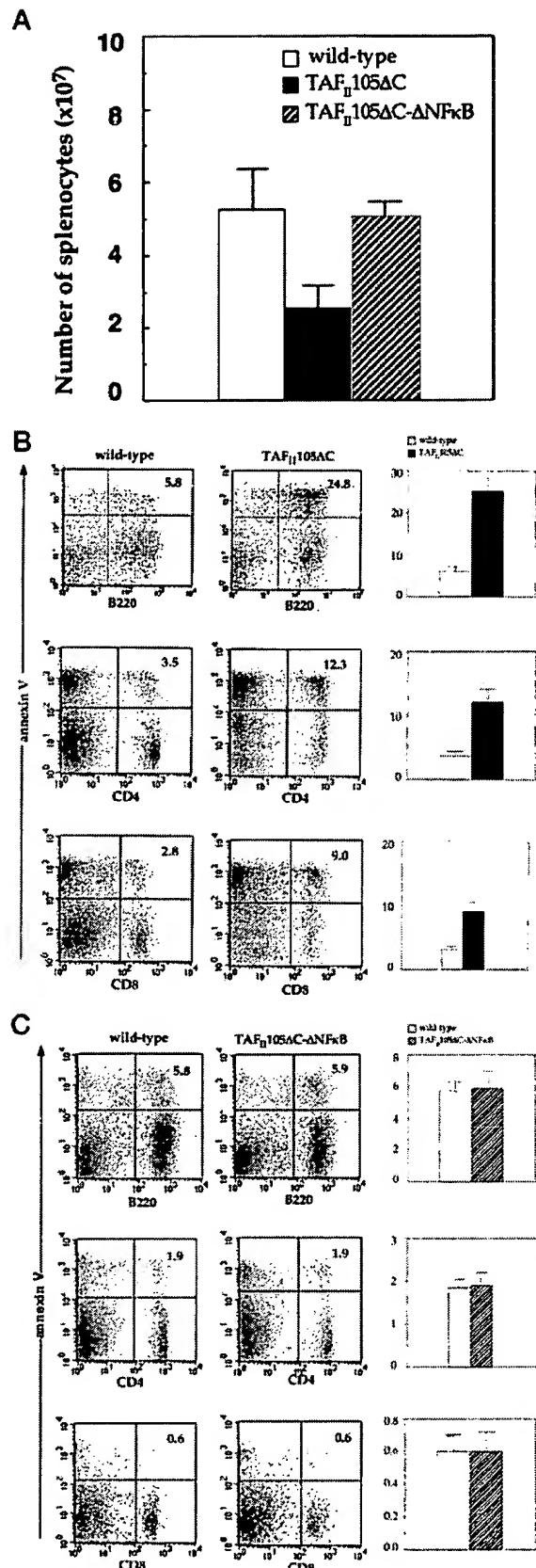


FIG. 4. Elevated apoptosis in splenocytes from TAF<sub>II</sub>105ΔC transgenic mice. *A*, reduced number of lymphocytes in spleens from TAF<sub>II</sub>105ΔC transgenic mice. Lymphocytes from wild-type and transgenic mice (lines 134 and Δ7, respectively) were prepared as described

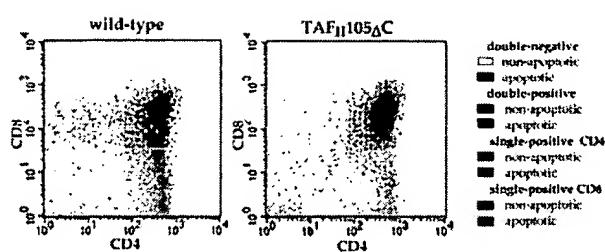
tions compared with wild-type animals (data not shown). However, the total number of lymphocytes in spleens from TAF<sub>II</sub>105ΔC transgenic mice was significantly lower than in spleens from wild-type or TAF<sub>II</sub>105ΔC-ΔNFκB animals (Fig. 4A). Taking into account the results of previous studies that showed an anti-apoptotic role for TAF<sub>II</sub>105 in cultured cells (15), we hypothesized that the reduction in lymphocyte number in transgenic mice might be caused by increased levels of apoptosis. To test this possibility, we performed FACS analysis of splenocytes from wild-type and transgenic mice labeled with anti-B220 (marker of B cells), anti-CD4 (marker of helper T lymphocytes), or anti-CD8 (marker of cytotoxic T lymphocytes) antibody together with annexin V, which serves as a marker of cells in early apoptosis. As shown in Fig. 4B, the percentage of apoptotic B cells (24.8 versus 5.8), helper T cells (12.3 versus 3.5), and cytotoxic T cells (9.0 versus 2.8) was significantly increased in splenocytes from TAF<sub>II</sub>105ΔC transgenic mice compared with wild-type animals. To investigate the possibility that inhibition of the anti-apoptotic response is associated with interaction with p65/RelA, a similar experiment was performed with transgenic animals expressing TAF<sub>II</sub>105ΔC-ΔNFκB. Remarkably, the number of apoptotic B and T cells in spleens from TAF<sub>II</sub>105ΔC-ΔNFκB mice was the same as in control mice (Fig. 4C).

To test whether TAF<sub>II</sub>105 is involved in the control of apoptosis also in thymus, we performed triple staining of thymocytes with CD4, CD8, and annexin V markers, followed by FACS analysis. By this analysis, we could monitor the relative number of apoptotic and non-apoptotic T cell populations: immature double-negative CD4<sup>-</sup>/CD8<sup>-</sup> T cells and double-positive CD4<sup>+</sup>/CD8<sup>+</sup> T cells and mature helper T cells (CD4<sup>+</sup>/CD8<sup>-</sup>) and cytotoxic T cells (CD4<sup>-</sup>/CD8<sup>+</sup>). We found a 3–4-fold increase in the proportion of apoptotic cells in all different thymocyte populations from TAF<sub>II</sub>105ΔC transgenic mice compared with wild-type animals (Fig. 5A). By contrast, the level of cell death of all T cell populations in TAF<sub>II</sub>105ΔC-ΔNFκB transgenic mice was similar to that in wild-type mice (Fig. 5B). Interestingly, the relative number of single-positive CD4<sup>+</sup> or CD8<sup>+</sup> T cells in TAF<sub>II</sub>105ΔC mice decreased by 2–3-fold, whereas no significant change in the proportion of double-positive and double-negative cell populations was observed despite the increase in cell death rates. These results suggest that TAF<sub>II</sub>105 may be important in early stages of T cell maturation common to both CD4<sup>+</sup> and CD8<sup>+</sup> cells.

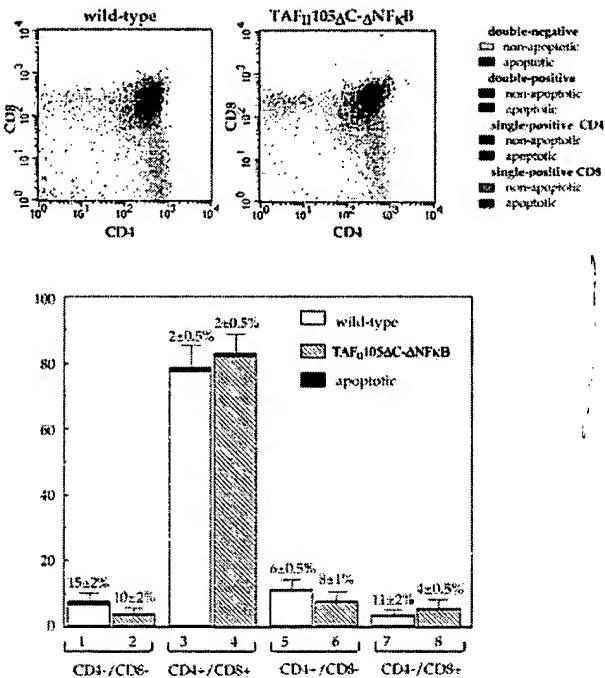
**Expression of the Anti-apoptotic Gene A20 Is Impaired in TAF<sub>II</sub>105ΔC Transgenic Mice**—Previously, we reported that the anti-apoptotic gene A20 is a transcriptional target of the substoichiometric TAF<sub>II</sub>105-TFIID complex (35). A20 is expressed at particularly high levels in lymphoid organs of mice, especially in spleen and thymus (37). To examine the possible effect of dominant-negative mutants of TAF<sub>II</sub>105 on expression of endogenous A20, we checked the level of A20 mRNA in wild-type and transgenic mice expressing TAF<sub>II</sub>105ΔC and TAF<sub>II</sub>105ΔC-ΔNFκB. RNA was extracted from the thymuses of wild-type and transgenic mice and used in quantitative RT-PCR employing a LightCycler with primers specific for either

under "Materials and Methods" and counted. Similar results were obtained in experiments with four other transgenic lines. *B*, elevated apoptosis in B lymphocytes (B220<sup>+</sup>), helper T lymphocytes (CD4<sup>+</sup>), and cytotoxic T lymphocytes (CD8<sup>+</sup>) from spleens of TAF<sub>II</sub>105ΔC transgenic mice (line 134). Lymphocytes were incubated with one of the antibodies against the cell markers (B220, CD4, or CD8) together with annexin V, which serves as a marker for apoptotic cells. The *upper right quadrants* represent the apoptotic populations of the cell types examined. *C*, an experiment similar to that described for *B* was performed with splenocytes from TAF<sub>II</sub>105ΔC-ΔNFκB transgenic mice (line Δ7). The *right panels* show the averages of results obtained from experiments with three transgenic lines.

A.

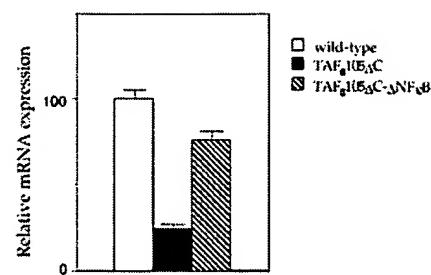


B.



**FIG. 5. Elevated apoptosis in thymocytes from TAF<sub>II</sub>105ΔC transgenic mice (line 134). A: upper panels,** T cell suspensions obtained from thymuses of wild-type and TAF<sub>II</sub>105ΔC transgenic mice were triple-stained with anti-CD4 and anti-CD8 antibodies and annexin V, followed by FACS analysis. The color index is shown on the right. **Lower panel,** the results of the same experiment are represented in a bar graph. The relative percentage of apoptotic cells in each population is shown above the bars and represents the average of three independent experiments. Similar results were obtained in experiments with two other transgenic lines. **B:** an experiment similar to that described for A was performed with thymocytes from TAF<sub>II</sub>105ΔC-ΔNFκB transgenic mice (line Δ7).

A.



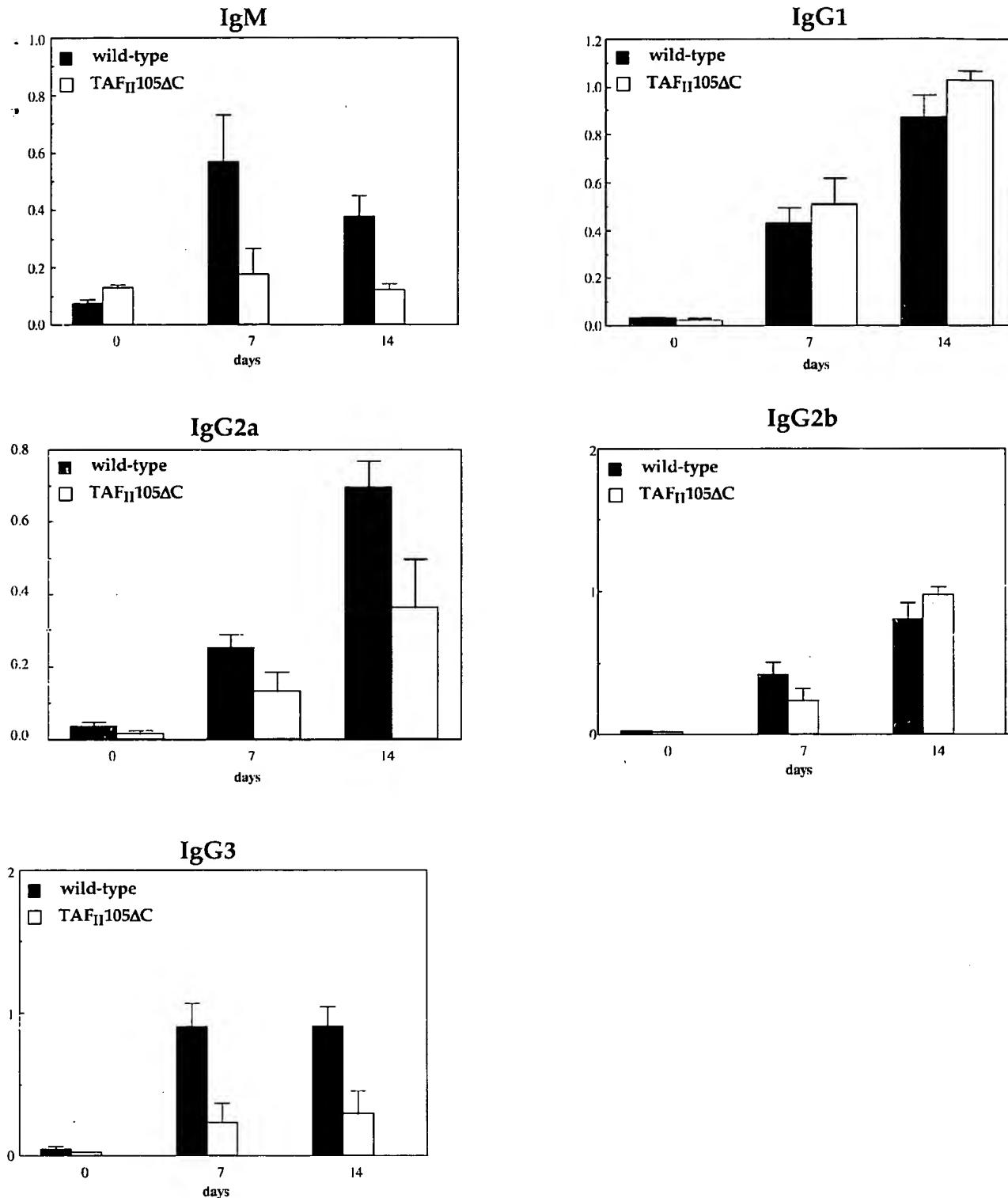
B.



**FIG. 6. A:** effect of TAF<sub>II</sub>105 mutants on A20 expression. RNAs from thymuses of wild-type and transgenic animals (line 134) were extracted (as described under "Materials and Methods") and used for real-time quantitative RT-PCR analysis using a LightCycler. The results are expressed as a percentage of the value of wild-type expression and normalized to the value of GAPDH. The identity of the PCR products was confirmed by running on agarose gel and staining with ethidium bromide. **B:** TAF<sub>II</sub>105 binds the A20 promoter *in vivo*. Daudi B cells were cross-linked *in vivo* with 1% formaldehyde. Soluble chromatin was prepared from these cells and used for immunoprecipitation with either control antibody (Ab; anti-hepatitis B virus core) or TAF<sub>II</sub>105-, TBP-, and p65/RelA-specific antibodies (chromatin immunoprecipitation assay). The immunoprecipitated DNAs were analyzed for the A20 promoter by PCR. To confirm the amplification of the A20 promoter in the immunoprecipitated samples, the PCR products were analyzed by Southern blotting.

the mouse A20 gene or the housekeeping gene GAPDH, which served as an internal control. Consistent with the fact that the A20 gene is regulated by NF-κB (38), we found that TAF<sub>II</sub>105ΔC inhibited A20 expression more efficiently than TAF<sub>II</sub>105ΔC lacking the major NF-κB-binding site (Fig. 6A). The partial inhibition of A20 expression observed in the TAF<sub>II</sub>105ΔC-ΔNFκB mutant may be explained by the presence of another NF-κB-binding site in this mutant (35). We also examined the effect of the transgenes on other survival genes such as *bcl-2* and *bcl-X<sub>L</sub>*, and found that both transgenes had little inhibitory effect on expression of these genes (data not shown), suggesting that these genes are not involved in the enhanced apoptosis in TAF<sub>II</sub>105ΔC mice. This finding is consistent with the notion that TAF<sub>II</sub>105 is required for transcription of a subset of NF-κB-regulated genes.

To examine the relevance of the inhibition of A20 gene transcription by TAF<sub>II</sub>105ΔC to the function of endogenous TAF<sub>II</sub>105 protein, we tested whether the A20 promoter is directly bound by the TAF<sub>II</sub>105-TFIID complex in living cells. For this purpose, DNA-protein complexes from the Daudi B cell line were cross-linked *in vivo* with formaldehyde, followed by soluble chromatin preparation and immunoprecipitation with either control antibodies or TAF<sub>II</sub>105-, TBP-, and p65/RelA-specific antibodies. The presence of the A20 promoter on the bound complexes was determined by PCR, followed by Southern blot analysis. As shown in Fig. 6B, the promoter of the A20 gene was specifically enriched by the anti-TAF<sub>II</sub>105, anti-TBP, and anti-p65/RelA antibodies, but not by the control antibodies. The enrichment of the promoter region by the anti-transcription factor antibodies was specific, as the coding region of the A20 gene was not immunoprecipitated by these antibodies (data not shown). These results provide strong evidence that the A20 promoter is directly bound and regulated by the substoichiometric



**FIG. 7. Selective inhibitory effect of the TAF<sub>II</sub>105ΔC transgenic mutant on the production of antigen-specific antibodies.** Mice were immunized with 50  $\mu$ g of KLH in complete Freund's adjuvant. The levels of anti-KLH Ig subclasses (at a serum dilution of 1:200) were determined by enzyme-linked immunosorbent assay 7 and 14 days after immunization. The results are expressed as  $A_{405}$  values.

metric TAF<sub>II</sub>105-TFIID complex *in vivo* as well as by NF- $\kappa$ B.

**Dominant-negative TAF<sub>II</sub>105 Has a Selective Inhibitory Effect on the Production of Antigen-specific Antibodies**—To examine the role of TAF<sub>II</sub>105 in the humoral immune response, we evaluated the capacity of the dominant-negative mutant to affect the production of immunoglobulins. To this end, we

measured the levels of antigen-specific antibodies in TAF<sub>II</sub>105ΔC transgenic and wild-type mice. The animals were immunized with KLH; and after 7 and 14 days, sera were checked by enzyme-linked immunosorbent assay using isotype-specific antibodies. The levels of anti-KLH IgM, IgG2a, and IgG3 in TAF<sub>II</sub>105ΔC transgenic mice were significantly lower

than in control animals, but no significant differences were found in the levels of IgG1 and IgG2b (Fig. 7). By contrast, the levels of IgM, IgG2a, and IgG3 were not changed in TAF<sub>II</sub>105ΔC-ΔNF<sub>κ</sub>B transgenic mice (data not shown). These results demonstrate the importance of TAF<sub>II</sub>105 in antibody production and isotype switching.

#### DISCUSSION

In this study, we have examined the function of TAF<sub>II</sub>105 in the context of the whole organism and determined the biological importance of a domain in TAF<sub>II</sub>105 involved in interaction with the NF-<sub>κ</sub>B protein p65/RelA *in vitro*. We generated and analyzed transgenic mice expressing dominant-negative mutants of TAF<sub>II</sub>105, which inhibit the function of TAF<sub>II</sub>105 and, most likely, also of TAF<sub>II</sub>105 homologs. Because we did not interfere with expression of the native protein, this strategy did not affect the stability of the endogenous TAF<sub>II</sub>105-TFIID complex. Furthermore, expression of different forms of dominant-negative mutants allowed us to examine the structure-function relationship involved in the mechanism of action of TAF<sub>II</sub>105 *in vivo*. We provide evidence that one of the domains within TAF<sub>II</sub>105 required for interaction with p65/RelA *in vitro* is crucial for lymphocyte survival and antibody production in the mouse. This notion is consistent with previous findings indicating that TAF<sub>II</sub>105ΔC-ΔNF<sub>κ</sub>B is a significantly less potent inhibitor of NF-<sub>κ</sub>B transcription activity than TAF<sub>II</sub>105ΔC (35) and strongly suggests that the C-terminal NF-<sub>κ</sub>B-binding site within TAF<sub>II</sub>105 is a functionally more important site than the N-terminal site. As NF-<sub>κ</sub>B has been established as a survival factor in lymphocytes (39–43), the finding that TAF<sub>II</sub>105 is important for activation of anti-apoptotic genes in B and T lymphocytes implies that it cooperates with p65/RelA in these cells to activate some of the NF-<sub>κ</sub>B target genes. The observation that the NF-<sub>κ</sub>B interaction domain is also required for IgM, IgG2a, and IgG3 isotype switching is also consistent with a recent finding showing that B cells lacking p65 exhibit a defect in switching to IgG3 (44). However, it has yet to be determined whether the effect of TAF<sub>II</sub>105ΔC expression on lymphocyte survival is intrinsic or involves contributions of other cell types.

Programmed cell death is of fundamental importance in the immune system and plays important roles in the control of the immune response and in lymphocyte development and cytotoxicity (reviewed in Refs. 45–47). In developing lymphocytes, cell death is the mechanism by which immune cells that recognize self-antigens are deleted. This process ensures the release of cells recognizing non-self-antigens into the periphery and determines the finite life span of terminally differentiated cells. Thus, apoptosis provides a flexible mechanism for controlling the composition and size of the mature cell population. It has been proposed that positive selection of thymocytes is a rescue from a default pathway of death in developing thymocytes (48–50). The TAF<sub>II</sub>105-NF-<sub>κ</sub>B complex may be required for the positive selection process during lymphocyte development and the immune response by translating upstream positive signals into activation of survival gene products. This idea is supported by our observation of a significant reduction in the relative numbers of single-positive mature T cells in TAF<sub>II</sub>105ΔC transgenic mice. This effect may be explained in part by the increased rate of apoptosis in the population of immature double-positive thymocytes, from which mature T cells develop by positive selection. However, given that the proportion of the immature double-positive T cells in the thymuses of TAF<sub>II</sub>105ΔC mice is not decreased despite high apoptotic rates, it is possible that TAF<sub>II</sub>105 is important for T cell maturation processes that are also apoptosis-independent.

The transgenic animals that we have obtained expressed

very low amounts of the dominant-negative proteins relative to the native endogenous protein. The accelerated apoptosis of lymphocytes observed despite low expression levels of the TAF<sub>II</sub>105ΔC transgene may result from the fact that the decision of lymphocytes to undergo apoptosis is dependent on the extent of positive and negative survival signals encountered by the cell. The amount of TAF<sub>II</sub>105ΔC protein in the lymphocytes of the transgenic animals may be sufficient to shift a delicate balance of pro- and anti-apoptotic signals toward apoptosis. Alternatively, cells expressing higher levels of the transgene are those that may have been eliminated by apoptosis. It is therefore likely that TAF<sub>II</sub>105 has additional functions that were not explored in this study, as inhibition of other functions of TAF<sub>II</sub>105 may require expression levels that are at least comparable to those of the native protein. Our inability to obtain transgenic animals expressing high levels of this protein and the decline of its expression over generations raise the possibility that the protein may be toxic to the animals.

Early biochemical studies have suggested that activator-TFIID interaction enhances the formation of a functional preinitiation complex (51–53). A question arising from this study is whether p65/RelA-TAF<sub>II</sub>105 interaction contributes to the assembly of the preinitiation complex by enhancing recruitment of the stoichiometric TAF<sub>II</sub>105-TFIID complex to the promoters of anti-apoptotic genes expressed in lymphocytes. Future experiments should address the molecular mechanism by which NF-<sub>κ</sub>B and its coactivators affect the transcription process *in vivo*.

**Acknowledgments**—We thank Dr. Ahuva Knyszynski, Dr. Tatyana Burakov, Judith Chermesh, Rafi Saka, and Dr. Ori Brenner (Weizmann Institute of Science Transgenic Facility) for generating and maintaining the transgenic mice and Dr. Sandra Moshonov for editing the manuscript and for technical assistance.

#### REFERENCES

1. Hoffmann, A., Oelgeschlager, T., and Roeder, R. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 8928–8935
2. Verrijzer, C. P., and Tjian, R. (1996) *Genes Dev.* **11**, 3020–3031
3. Deleted in proof
4. Chalkley, G. E., and Verrijzer, C. P. (1999) *EMBO J.* **18**, 4835–4845
5. Kauffmann, L., and Smale, S. (1994) *Genes Dev.* **8**, 821–829
6. Martinez, E., Chiang, C.-M., Ge, H., and Roeder, R. G. (1994) *EMBO J.* **13**, 3115–3126
7. Smale, S. T. (1997) *Biochim. Biophys. Acta* **1351**, 73–88
8. Verrijzer, C. P., Y. K. Chen, J. L., and Tjian, R. (1994) *Science* **264**, 933–941Y. K.
9. Albright, S. R., and Tjian, R. (2000) *Gene (Amst.)* **242**, 1–13
10. Bertolotti, A., Bell, B., and Tora, L. (1999) *Oncogene* **18**, 8000–8010
11. Lavigne, A. C., Mengus, G., Gangloff, Y. G., Wurtz, J. M., and Davidson, I. (1999) *Mol. Cell. Biol.* **19**, 5486–5494
12. May, M., Mengus, G., Lavigne, A. C., Chambon, P., and Davidson, I. (1996) *EMBO J.* **15**, 3093–3104
13. Mengus, G., May, M., Carre, L., Chambon, P., and Davidson, I. (1997) *Genes Dev.* **11**, 1381–1395
14. Wolstein, O., Silkov, A., Revach, M., and Dikstein, R. (2000) *J. Biol. Chem.* **275**, 16459–16465
15. Yamit-Hezi, A., and Dikstein, R. (1998) *EMBO J.* **17**, 5161–5169
16. Deleted in proof
17. Pham, A. D., Muller, S., and Sauer, F. (1999) *Mech. Dev.* **84**, 3–16
18. Zhou, J., Zwicker, J., Szymanski, P., Levine, M., and Tjian, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13483–13488
19. Apone, L. M., Virbasius, C. M., Reese, J. C., and Green, M. R. (1996) *Genes Dev.* **10**, 2368–2380
20. Apone, L. M., Virbasius, C. A., Holstege, F. C., Wang, J., Young, R. A., and Green, M. R. (1998) *Mol. Cell* **2**, 653–661
21. Metzger, D., Scheer, E., Soldatov, A., and Tora, L. (1999) *EMBO J.* **18**, 4823–4834
22. Michel, B., Komarnitsky, P., and Buratowski, S. (1998) *Mol. Cell* **2**, 663–673
23. Mootaderi, Z., Bai, Y., Poon, D., Weil, P. A., and Struhl, K. (1996) *Nature* **383**, 188–191
24. Mootaderi, Z., Keaveney, M., and Struhl, K. (1998) *Mol. Cell* **2**, 675–682
25. Natarajan, K., Jackson, B. M., Rhee, E., and Hinnebusch, A. G. (1998) *Mol. Cell* **2**, 683–692
26. Komarnitsky, P. B., Michel B., and Buratowski, S. (1999) *Genes Dev.* **13**, 2484–2489
27. Sanders, S. L., Klebalow, E. R., and Weil, P. A. (1999) *J. Biol. Chem.* **274**, 18847–18850
28. Walker, S. S., Reese, J. C., Apone, L. M., and Green, M. R. (1996) *Nature* **383**, 185–188
29. Reese, J. C., Zhang, Z., and Kurpad, H. (2000) *J. Biol. Chem.* **275**, 17391–17398

30. Grant, P. A., Schielz, D., Pray-Grant, M. G., Steger, D. J., Reese, J. C., Yates, R., Jr., and Workman, J. L. (1998) *Cell* **94**, 45–53
31. Ogryzko, V. V., Kotani, T., Zhang, X., Schlitz, R. L., Howard, T., Yang, X. J., Howard, B. H., Qin, J., and Nakatani, Y. (1998) *Cell* **94**, 35–44
32. Dikstein, R., Zhou, S., and Tjian, R. (1996) *Cell* **87**, 137–146
33. Freiman, R. N., Albright, S. R., Zheng, S., Sha, W., C., Hammer, R. E., and Tjian, R. (2001) *Science* **293**, 2084–2087
34. Furukawa, T., and Tanese, N. (2000) *J. Biol. Chem.* **275**, 29847–29856
35. Yamin-Hezi, A., Nir, S., Wolstein O., and Dikstein, R. (2000) *J. Biol. Chem.* **275**, 18180–18187
36. van Lohuizen, M., Verbeek, S., Krimpenfort, P., Domen, J., Saris, C., Radaszkiewicz, T., and Berns, A. (1989) *Cell* **56**, 673–682
37. Tewari, M., Wolf, F., Seldin M., O'Shea, K., Dixit, V., and Turka, L. (1995) *J. Immunol.* **154**, 1699–1706
38. Krikos, A., Laherty, C. D., and Dixit, V. M. (1992) *J. Biol. Chem.* **267**, 17971–17976
39. Barkett, M., and Gilmore, T. (1999) *Oncogene* **18**, 6910–6924
40. de Martin, R., Schmidt, J. A., and Hofer-Warbinek, R. (1999) *Mutat. Res.* **437**, 231–243
41. Foo, S. Y., and Nolan, G. P. (1999) *Trends Genet.* **15**, 229–235
42. Grossmann, M., O'Reilly, L. A., Gugasyan, R., Strasser, A., Adams, J. M., and Gerondakis, S. (2000) *EMBO J.* **19**, 6351–6360
43. Huesmann, M., Scott, B., Kieslow, P., and von Boehmer, H. (1991) *Cell* **68**, 533–542
44. Horwitz, B. H., Zelazowski, P., Shen, Y., Wolcott, K. M., Scott, M. L., Baltimore, D., and Snapper, C. M. (1999) *J. Immunol.* **161**, 1941–1946
45. Cohen, J. J., Duke, R. C., Fadok V. A., and Sellins, K. S. (1992) *Annu. Rev. Immunol.* **10**, 267–293
46. Williams, G. T. (1994) *Biochem. Cell Biol.* **72**, 447–450
47. Wyllie, A. H. (1987) *Int. Rev. Cytol.* **17**, 755–785
48. Egerton, M., Scollay, R., and Shortman, K. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2579–2582
49. Deleted in proof
50. Surh, C. D., and Sprent, J. (1994) *Nature* **372**, 100–103
51. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
52. Orlando, V., Strutt, H., and Paro, R. (1997) *Methods: A Companion to Methods in Enzymology*, Vol. 11, pp. 205–214, Academic Press, Inc., San Diego, CA
53. Roeder, R. G. (1991) *Trends Biochem. Sci.* **16**, 402–408